Novel probiotics isolated from fish gut microbiota for improving plant feedstuff utilization, and gut health of carnivorous fish

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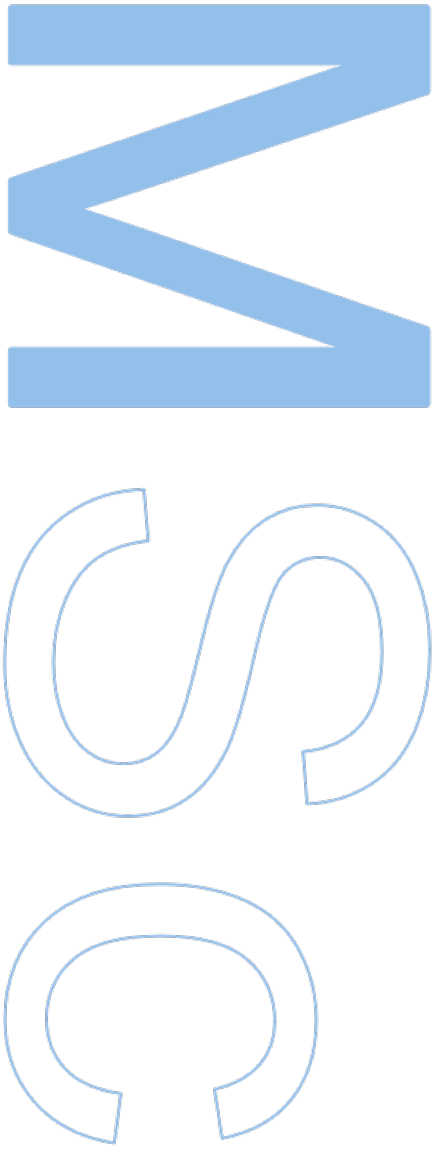

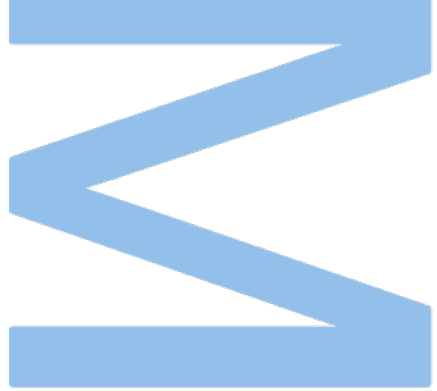

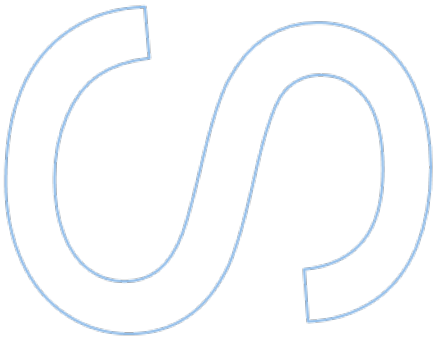


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You've got to find yourself first. Everything else'll follow.

Charles de Lint - Dreams Underfoot

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Abstract

The demographic growth has generated an ever-increasing demand for animal protein. As a result, the aquaculture industry has emerged as a critical source of aquatic animal protein, accounting for nearly 50% of total aquatic organism production in 2020. This growth has intensified the search for suitable aquafeeds ingredients required to sustain the produced expanding biomass. Aquafeed producers are increasingly turning to plant-based ingredients as an alternative to fishmeal, which often exhibits high price variability and supply constraints.

The adoption of plant feedstuffs in aquafeeds correlates with the introduction of anti-nutritional factors that can potentially harm fish health and compromise their overall performance. Non-starch polysaccharides (NSPs) represent a category of these anti-nutritional factors, that besides not being digestible by the fish, may also reduce the availability of nutrients present in diets, alter fish gut anatomy, and decrease growth performance.

To enhance the utilization of plant-based diets, exogenous enzymes are commonly utilized. An alternative approach involves the incorporation of probiotics with carbohydrolytic capabilities, that may also potentially improve feed efficiency, enhance gut health, and mitigate oxidative stress. Among the various probiotics used in aquaculture, *Bacillus* spp. stand out due to their ability to form spores, making long-term storage possible, as well as large-scale industrial production. Moreover, spores can survive high temperatures and a wide range of pH levels, thus being able to endure both the aquafeed manufacturing process and the digestive tract transit.

In a study by Serra et al. (2019), a strain of *Bacillus subtilis* (Fish Isolate 99 - FI99) was isolated from the gut of European sea bass (*Dicentrarchus labrax*) and demonstrated, *in vitro*, carbohydrolytic activity. When FI99 was incorporated into plant-based diets at a concentration of 1 x 10^9 spores per kilogram of feed, growth performance in European sea bass was promoted with a tendency to reach levels similar to the fishmeal-based diet.

This project is a follow-up of the previously study, aiming to fine-tune the optimal level of FI99 incorporation and to compare its effectiveness with that of commercial exogenous carbohydrases, to enhance the growth performance and intestinal health of European sea bass.

Five isoproteic (48%) and isolipidic (18%) diets were formulated. Animal protein in the diets was restricted to 15% in order to obtain challenging plant-based diets. Diets were supplemented with increasing levels of FI99 as follows: 1 x 10^9 CFU kg^{-1} (D1), 7 x 10^9 CFU kg^{-1} (D2), 4 x 10^{10} CFU kg^{-1} (D3). A diet without probiotic inclusion was used as a control (CTR) and an enzymatic cocktail (Natugrain™) containing endo -1, 4-β-xylanase (5600 TXU g^{-1}) and endo -1, 4-β-glucanase (2500 TGU g^{-1}) was supplemented (0.04%) in a diet (EXO) similar to the control.

No significant differences were observed in the growth performance and in the histomorphology of the distal intestine of the fish. Digestive enzymes activities were not affected by either probiotic or Natugrain™ inclusions.

No differences were found between the CTR diet and the experimental diets in dry matter and protein apparent digestibility coefficients (ADC). Dry matter ADC was higher in EXO diet compared to D1 and D3 diets, but similar to CTR and D2 diets. Energy ADC of diets was positively affected by exoenzyme inclusion. Probiotic inclusions diminished diets energy ADC.

Catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) activities in the intestine, were not affected by either inclusion of FI99 or exoenzyme. Superoxidase dismutase (SOD) activity was positively affected by incorporation of the exogenous enzyme cocktail. The inclusion of FI99 did not influence SOD activity compared to CTR. Diets D1 and D2 show SOD activity similar to EXO. In contrast, D3 demonstrates significantly lower activity values compared to both EXO and D1. Lipid peroxidation of the intestine was not affected either by probiotic or exoenzyme supplementation.

In conclusion, both FI99 and Natugrain™ had no significant effect on the performance and gut health of the fish.

Keywords: Aquaculture, *Bacillus spp.*, Exogenous enzymes, Non-starch polysaccharides, Probiotics

Resumo

O crescimento demográfico conduziu a um aumento na procura de proteína animal de origem aquática, surgindo a indústria da aquacultura como uma fonte fundamental dessa proteína. Em 2020, a aquacultura contribuiu com cerca de 50% da produção total de organismos aquáticos. Este crescimento intensificou a procura por ingredientes adequados para incluir nas rações, de modo a sustentar a biomassa produzida. Os produtores de rações têm recorrido a matérias-primas vegetais como alternativa à farinha de peixe, que apresenta uma elevada variabilidade de preço e restrições de fornecimento.

A incorporação de matérias-primas vegetais tem como principal inconveniente a presença de fatores anti-nutricionais que podem prejudicar a saúde dos peixes e comprometer o seu crescimento. Os polissacarídeos não amiláceos (NSPs) são um exemplo de fatores anti-nutricionais. Estes não são digeridos pelos peixes, podendo reduzir a disponibilidade de nutrientes presentes nas dietas, alterar a anatomia do intestino e diminuir o crescimento dos peixes.

Enzimas exógenas são comumente utilizadas para melhorar a utilização de rações à base de matérias-primas vegetais. Uma abordagem alternativa envolve a incorporação de probióticos com capacidades carbohidrolíticas, melhorando a eficiência alimentar, promovendo a saúde intestinal e mitigando o stress oxidativo nos peixes. Entre os probióticos utilizados em aquacultura, *Bacillus* spp., surgem como uma alternativa promissora devido à sua capacidade de formarem esporos, facilitando o armazenamento e a produção industrial em larga escala. Os esporos resistem a elevadas temperaturas e a uma ampla gama de valores de pH, sendo capazes de resistir tanto ao processo de fabricação de rações como às condições do trato digestivo dos peixes.

Num estudo conduzido por Serra et al. (2019), foi isolado do intestino do Robalo (*Dicentrarchus labrax*), uma estirpe de *Bacillus subtilis* (Isolado de Peixe 99 - FI99) que demonstrou *in vitro* possuir atividade carbohidrolítica. Quando esporos de FI99 foram incorporados em dietas à base de matérias-primas vegetais numa concentração de 1 x 10^9 esporos kg^{-1} de alimento, observou-se um incremento do crescimento do Robalo, com valores próximos dos obtidos com uma dieta à base de farinha de peixe.

Este projeto é uma continuação do estudo anterior, propondo-se ajustar o nível ótimo de incorporação do FI99 em dietas para Robalo bem como comparar a eficácia do FI99 relativamente a carbohidrases comerciais, de forma a melhorar tanto o desempenho do crescimento como a saúde intestinal do Robalo.

Formularam-se cinco dietas isoproteicas (48%) e isolipídicas (18%). A proteína animal das dietas foi restrita a 15% de forma a obter dietas desafiantes à base de matérias-primas vegetais. As dietas foram suplementadas com concentrações crescentes do FI99: 1 x 10^9 CFU kg^{-1} (D1), 7 x 10^9 CFU kg^{-1} (D2) e 4 x 10^{10} CFU kg^{-1} (D3). Uma dieta sem inclusão de probióticos foi utilizada como controlo (CTR) e a uma dieta semelhante à CTR foi adicionado (0,4%) um cocktail enzimático comercial (Natugrain™) contendo endo-1,4-β-xilanase (5600 TXU g^{-1}) e endo-1,4-β-glucanase (2500 TGU g^{-1}) (EXO).

Não foram observadas diferenças significativas nem no crescimento, nem na histomorfologia do intestino distal dos peixes. As atividades das enzimas digestivas não foram afetadas pela inclusão do probiótico ou do cocktail enzimático.

Não foram encontradas diferenças significativas entre a dieta CTR e as dietas experimentais nos coeficientes de digestibilidade aparente (ADC) da matéria seca e da proteína. O ADC da matéria seca foi maior na dieta EXO em comparação com as dietas D1 e D3, mas semelhante às dietas CTR e D2. O ADC da energia das dietas foi positivamente afetado pela inclusão do cocktail enzimático. A inclusão de diferentes níveis de FI99 diminuíram o ADC de energia.

A atividade da catálase (CAT), da glutationa peroxidase (GPX) e da glutationa redutase (GR) no intestino não foi afetada pela inclusão de FI99 ou do cocktail enzimático. A atividade da superóxido dismutase (SOD) foi positivamente afetada pela incorporação do cocktail enzimático. As inclusões de FI99 não tiveram influência na atividade da SOD comparado com CTR. Dietas D1 e D2 demonstram atividade da SOD similares a EXO. Contrariamente D3 demonstra valores de atividade significativamente inferiores a tanto EXO e D1. A peroxidação lipídica do intestino não foi afetada nem pela suplementação de probióticos nem de enzima exógena.

Em conclusão, tanto FI99 como Natugrain™ não tiveram efeito significativo na performance e na saúde intestinal dos peixes.

Palavras-chave: Aquacultura, *Bacillus spp.*, Enzimas exógenas, Polissacarídeos não amiláceos, Probióticos

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ADC – Apparent digestibility coefficients

B&W – Bott & Wilson

CAT – Catalase

CFU – Colony Forming Units

DI – Distal Intestine

DSM – Difco Sporulation Medium

ESB – European sea bass

FI99 – Fish Isolate 99

FM – Fish Meal

GPX – Glutathione peroxidase

GR – Glutathione reductase

GSSG – Oxidized glutathione

LPO – Lipid Peroxidation

LB – Luria-Bertani

MDA – Malondialdehyde

NSP – Non-Starch Polysaccharide

PBS – Phosphate-buffered Saline

PF – Plant Feedstuff

RAS – Recirculatory Aquaculture System

ROS – Reactive Oxygen Species

SDS – Sodium Dodecyl Sulphate

SOD – Superoxidase dismutase

TAP – Total Alkaline Protease

TCA – Trichloroacetic acid

TBARS – Thiobarbituric acid reacting substances

XOS – Xylooligosaccharides

1. Introduction

1.1. Aquaculture: Brief categorization and overview

With the steady increase in the global human population, the food demand will also increase, as dietary trends seem to have a propensity toward resource-intensive foods (Gephart et al., 2021; Mancosu et al., 2015). Indeed the increase in global human population and the general rise in incomes seem to reflect the demand for animal protein in the form of milk, meat, and products from the aquatic environment, such as aquatic foods (FAO, 2022; Schubel & Thompson, 2019). According to FAO (2022), in 2019, foods from the aquatic environment represented 17% of the source of animal proteins, and 7% of all proteins.

Aquaculture can be defined as the practice of collecting, nourishing, reproducing, and safeguarding aquatic resources to support various human endeavours, whether they are commercial, recreational, or for public use (Mizuta et al., 2022). It is a fast-growing industry that, if done sustainably, can reduce the pressure on fishery resources and fish stocks, offer new job opportunities, and reduce aquatic food prices (Frankic & Hershner, 2003). In 2020, the global production of aquatic organisms was 178 million tonnes, with aquaculture contributing to 49% of this mass (equivalent to 88 million tonnes) (FAO, 2022) (Figure 1). Thus, the aquaculture sector and its reared biomass are expanding, recording a 2.6% growth in 2019/20, although at a slower rate than the one previously observed in 2018/19 (3.3%). This drop in production can partially be explained due to various issues caused by COVID-19, such as sanitary measures, lack of transportation and marketing, and reduced availability of different inputs (e.g. workers and supplies) (FAO, 2022). Nevertheless, aquaculture still proves to be a fundamental provider of animal protein in a world where its needs and demand are increasing, and it is an industry that is expected to continue growing in the near future (Boyd et al., 2022; FAO, 2022).

Aquaculture production systems can be divided into three major groups, extensive, semi-intensive and intensive systems, depending on the amount of human interaction that the system needs, type of feed utilized, and biomass of the reared animals.

Extensive systems require little to no human interaction and are normally comprised of nets, ponds, or cages where animals can be farmed. The reared animals are fed with natural food, or not fed at all. For example, in ponds, there is no additional feed, as the ecosystem inside the pond provides all the feed for the reared animals. These are the systems with the least production of the three groups. The definition of semi-intensive

systems can vary depending on the author, but most regard it as a combination/in-between of extensive and intensive systems characteristics. These systems have some degree of human action, for example, supplementary feeding, but mainly rely on natural food sources. Fertilization of the ecosystem present in the aquaculture system may be carried out but is not mandatory. Mechanical water treatments are usually not done, but water changes are utilized to renew the water quality.

Intensive aquaculture systems have a controlled input of water, feeding, and stocking. They rely heavily on the utilization of different water treatment options, to maintain good water quality, since feeding and stocking rates are maximized to optimize production. The basic controlled water parameters levels are oxygen, carbon dioxide, solids control (both organic and non-organic), as well as nitrogen-based compounds. The productivity of a system seems to increase according to its intensity, and the efficiency of water usage seems to follow this trend as well. In fact, the intensification of an aquaculture system may provide good usage of water resources, possibly compensating for water shortages, thus being a possible mean of water saving (Ahmed et al., 2017; Oddsson, 2020).

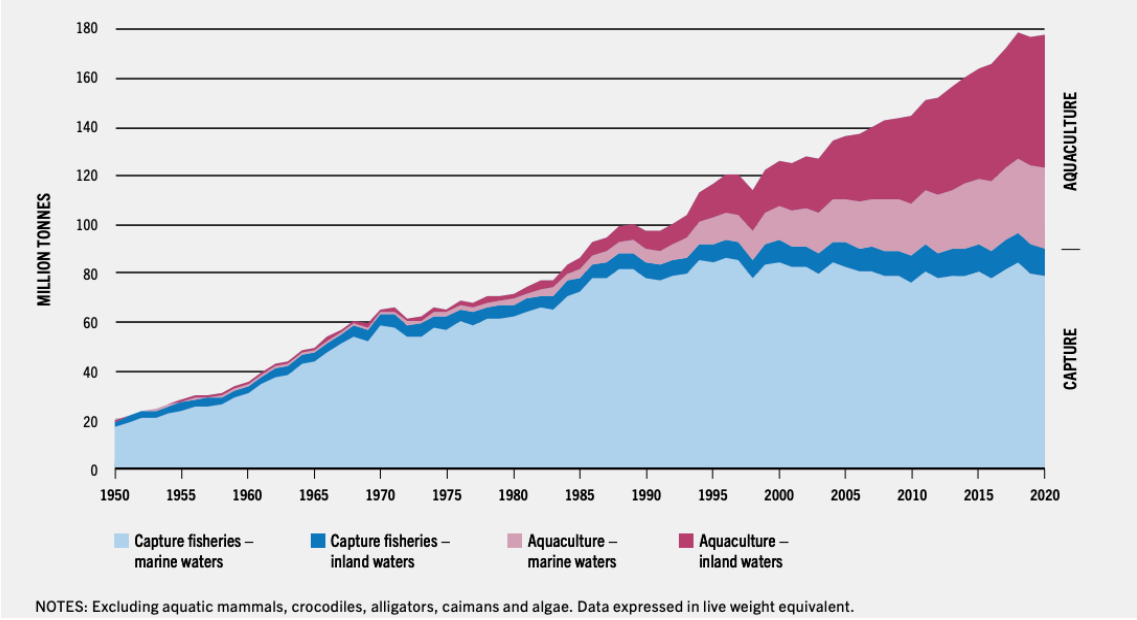

Figure 1. Global fisheries and aquaculture production (FAO, 2022).

1.2. European sea bass (*Dicentrarchus labrax*) and its production

The model species that was utilized in this study was the European sea bass (ESB) (*Dicentrarchus labrax)*, as this species has a key role in Mediterranean aquaculture, capture, and recreational activities. Overall, the Mediterranean accounts for 94% of all

ESB production (Vandeputte et al., 2019). Furthermore, the aquaculture of ESB accounts for 28.2% of total farm fish production in the Mediterranean, being the third most produced species after Seabream (*Sparus aurata*) and Rainbow Trout (*Oncorhynchus mykiss*). The latter species is reared in freshwater, solidifying the importance of ESB in marine aquaculture (Cantillo et al., 2022).

Relating to aquaculture production in Portugal, significant progress didn't occur until the 1990s, with initial importance placed on the rearing of Seabream and ESB (Rocha et al., 2022). In 2021, marine and transition fish production increased 27%, totalling to 44.2% (amounting to 7 912 tonnes) of all aquaculture production in Portugal, in comparison to 2020. ESB aquaculture production grew 5.5%, corresponding to 954 tonnes, comparing to 2020 (INE, 2022).

The aquaculture production of this species is typically carried out in two main steps; i) hatcheries/pre-growing, which produce mainly small fish from 1 to 20 g carried out in three to eight months; ii) on growing phase to the commercial size (250 - 450 g), taking up to more than a year to reach to this state (12-20 months) (Vandeputte et al., 2019).

This species is described as a demersal marine coastal fish (< 100 m of depth), ranging from the northeastern Atlantic Ocean to the Mediterranean and the Black Sea. It is a euryhaline species, tolerating salinities from 0 to 40 ppt, and eurythermal, tolerating temperatures from 2 to 32ºC (Vandeputte et al., 2019). Despite this temperature toleration range, juveniles seem to cease growth at 11-15ºC and have a growth spurt at 22-25ºC (Person-Le Ruyet et al., 2004).

Sexual maturation is different according to sex, and males seem to mature prematurely compared to females (2-3 years vs. 3-4 years) (Vandeputte et al., 2019). It appears to exist sexual dimorphism as females tend to be larger than males. Growth is diminished in males during puberty, thus being a limitation in the production of this species (Zanuy et al., 2001). Reproduction can happen in two different periods according to the location: December to March in the Mediterranean, and March to June in the Atlantic (Vandeputte et al., 2019).

ESB is a carnivorous species and thus, has high dietary protein requirements (45-48%), which are stable even with water temperature changes (Oliva-Teles, 2000; Peres & Oliva-Teles, 2013).

Regarding dietary lipid content, ESB has optimal growth when it has lipid levels between 12 and 24%. However, the inclusion of 30% lipids in the diets causes a great decline in growth performance (Oliva-Teles, 2000).

This species can tolerate high amounts of plant feedstuffs in diets, as it has been described that dietary fiber levels ranging from 3 - 100 g kg^{-1} have no negative effects on the performance of ESB (Kousoulaki et al., 2015).

1.3. Fishmeal and plant feedstuffs

Fishmeal (FM) is a high-protein source made by milling or grinding whole fish or fish parts. It is considered the ideal source of protein in aquafeeds due to its balanced amino acids profile, high digestibility, and high palatability, while, at the same time, offering no anti-nutritional factors (FAO, 2022).

Until very recently, this ingredient was traditionally used as aquafeeds main protein source. However, its production is highly dependent on the catch of small pelagic fish (anchoveta, sardine, mackerel, amongst others). The unpredictability of fish stocks due to natural events like El Niño-Southern Oscillation has led to a decrease in FM availability. Thus, the limited supply of FM and increasing aquaculture production are causing economic pressure on this ingredient leading to its elevated costs (FAO, 2022; Naylor et al., 2021; Tacon & Metian, 2008). Therefore, reducing the incorporation of FM in aquaculture feeds is crucial for the sustainable development of the intensive aquaculture industry (Oliva-Teles et al., 2015).

In this sense, efforts have been made to decrease the excessive use of aquatic animal protein sources in aquafeeds. As an alternative, various ingredients have been employed to serve as protein sources such as animal by-products, processed animal protein, micro and macroalgae, and plant feedstuff (PF) (Hua et al., 2019).

PF have become the main substitute for FM in aquafeeds (Olsen & Hasan, 2012), as generally these sources are more economically available and more sustainable than FM (Salin et al., 2018). PF can comprise soybean, which is regarded as both economically cheap and nutritional ingredient, corn, that is mainly produced as an energy source to livestock, wheat, rapeseed, amongst others.

Protein levels in these ingredients are inconstant (for example soybean displays ± 44% crude protein, corn gluten contains at least 60% crude protein, and wheat contains less than 12% protein) (Gatlin III et al., 2007; Oliva-Teles et al., 2015).

Furthermore, PF are also sources of different carbohydrates (Gatlin III et al., 2007). Although fish do not have any carbohydrate requirements, their inclusion in proper levels, may induce protein and lipid sparing effects, thus increasing protein and lipid retention utilized in growth and tissue maintenance (Villasante et al., 2019).

Despite PF higher availability and economical sustainability comparing to FM, these protein sources have some disadvantages. Vegetable ingredients may vastly differ in amino acidic (Kaushik & Hemre, 2008; Kaushik & Seiliez, 2010), vitamin and mineral profiles, and single use of one of these ingredients may not cover fish requirements (Salin et al., 2018; Torrecillas et al., 2017).

High dietary inclusion levels of PF may also cause adverse effects on the growth of different fish, due to the presence of anti-nutritional factors that may also have a harmful impact on fish gut health and disease resistance (Salin et al., 2018; Torrecillas et al., 2017). This effect is seen, for example, in salmonids, as the replacement of >33% of FM with soybean can lead to the development of pathological changes in the fish gut (Salin et al., 2018).

Other protein sources are utilized as partial substitutes of FM, but nevertheless PF is the most utilized in the industry (Salin et al., 2018).

1.4. Non-Starch Polysaccharides

Non-starch polysaccharides (NSPs) are one group of anti-nutritional factors present in PF. These polysaccharides such as cellulose, pectins and β-glucans, can compose up to 90% of plant cell walls (Kumar et al., 2012; Sinha et al., 2011). NSPs monomers are mainly linked by β-glycosidic bonds in opposition to starch, whose monomers are linked by α-glycosidic bonds. While fish may have several enzymes, with different levels and activities, with the capability to hydrolyze α-glycosidic bonds (e.g. α-amylase, α-glucosidase, and oligo-1-6-glucosidase), they lack significant levels of β-glucanase or β-xylanase, that are indispensable to digest NSPs bonds (Sinha et al., 2011).

NSPs are considered anti-nutritional factors, as they can reduce the availability of nutrients, affect the mixing of digestive enzymes and subtracts, and diminish the overall nutrient and energy digestion and absorption, by augmenting the digesta viscosity (Leenhouwers et al., 2006; Sinha et al., 2011).

Regarding the decrease in nutrient absorption, Atlantic salmon (*Salmo salar*) fed with high levels (200 g kg^{-1}) of soybean meal, a PF rich in NSPs (21.4 g kg^{-1}), showed an

impairment of protein digestibility compared with diets with pea protein concentrate that had minor levels of NSPs (12.1-14.0 g kg^{-1}) (Øverland et al., 2009). Protein and lipid digestion and absorption seem to be negatively affected, in different fish species, fed with different PF sources (Amirkolaie et al., 2005; Deng et al., 2010; Leenhouwers et al., 2007; Øverland et al., 2009), cumulating in a decreased of fish growth performance (Deng et al., 2021; Jiang et al., 2022).

NSPs can alter, either increasing or diminishing, the transit time of the digesta in the intestine, thus affecting nutrient digestibility time and restricting nutrient utilization (Krogdahl et al., 2005).

Additionally, long-term consumption of NSPs may alter fish's gut anatomy, histology, and development. These changes include: i) an increase in the size and length of the digestive organs; and/or ii) shortening and fusion of the distal intestinal folds, resulting in a decrease in nutrient absorption and digestion (Haidar et al., 2016; Kraugerud et al., 2007; Sinha et al., 2011).

Despite these negatives effects of NSPs, studies have shown that at small levels, generally less than 2%, these polysaccharides, namely β-glucan, pectin, and fructo-oligosaccharides, may have positive effects in fish including: i) increased growth, due to alteration of sugar and lipid levels in the blood; ii) modulation of glucose absorption, reducing hyperglycemia; and iii) body composition, as diets with low levels of NSPs have been associated with higher whole-body protein and lower whole-body lipid contents (Wang et al., 2022).

The gut microbiota has a significant role in the fermentation and digestion of plant materials in fish (Clements et al., 2014). Hence, intestinal microbiota can hydrolyze NSPs, and the sub-products may impact the host's metabolism and immunity (Sinha et al., 2011; Wang et al., 2022).

In a previous work, feeding ESB with diets with high levels of NSPs led to the establishment of intestinal microbiota taxa (e.g., *Bacillaceae*) capable of hydrolysing NSPs (Serra et al., 2021).

These microorganisms may benefit the host by increasing growth and improving the immune system and gastrointestinal histomorphology. Thus, the inclusion of the previously mentioned organisms could be an alternative to dietary commercial carbohydrases supplementation (Serra et al., 2019), as the addition of these enzymes

to aquafeed does not necessarily mean an increased growth performance in different species, such as rainbow trout (Dalsgaard et al., 2012), and Nile tilapia (*Oreochromis niloticus*) (Yigit & Olmez, 2011).

1.5. Fish gut microbiota

Microbiota can be described as the collection of living microorganisms present in a defined environment (Berg et al., 2020). These microorganisms have the potential to form commensal or mutual relationships with the host (Romero et al., 2014).

The gut microbiota can be divided into two major groups: autochthonous bacteria, which are considered permanent and intimately associated to host gut mucosa thus, being able to colonize the host gut and form the core of the microbiota, and allochthonous bacteria, whose permanence time is considerably lower, being considered “incidental visitors” and associated normally with the digesta (Banerjee & Ray, 2017; Ray et al., 2012; Ringø et al., 1995; Romero et al., 2014).

These communities present in the fish gut display fundamental roles in metabolic and digestive functions, development of the digestive apparatus, and immune responses, therefore having a relevant role in the host health (Ringø et al., 2022).

The initial colonization of the microbiota in fish larvae is mainly dependent of the microbiota of the eggs, the larval rearing water, and the live feed (Wang et al., 2018).

Although the gut microbiota is formed at early larval stages, it can be later modulated depending on diet composition (Wang et al., 2018). The composition of the microbiota can be influenced by other factors originating either from within the organism or from its external environment (e.g. nutritional status, rearing water, among others), interfering with fish health status (Banerjee & Ray, 2017; Ringø et al., 2022).

Thus, as previously mentioned, it is possible to induce the modulation of fish gut microbiota through a selective pressure of PF diets, in order to isolate probiotic bacteria with the capability of utilizing NSPs as substrate, as shown in Serra et al. (2019).

1.6. Probiotics and *Bacillus* spp.

Probiotics are living microorganisms, bacteria or yeast, that when administered in adequate amounts may provide health benefits to the host (Boyd et al., 2020; Senok et

al., 2005; Serra et al., 2019). Generally, probiotics can enhance the host's gastrointestinal development, aid the digestion of nutrients, improve disease resistance, and adjuvant the immunological response (Boyd et al., 2020). Furthermore, the health status of the organism can be influenced as probiotics may inhibit pathogens through the production of antagonistic compounds, reduction of available attachment sites, engaging in competition for nutrients and/or modulation of enzymatic activities of pathogens (Kesarcodi-Watson et al., 2008).

Probiotics enzymes may increase the host digestive enzymatic activity, consequently improving feed digestibility and utilization (Kesarcodi-Watson et al., 2008; Yilmaz et al., 2022). These bacteria and yeast may also improve fish growth, water quality by reducing the amount of antibiotic usage, and aiding in the decomposition of organic matter (Nayak, 2010), thus contributing to aquaculture's sustainability.

Regarding the gut microbiota, probiotics could favour the development and establishment of beneficial intestinal microorganisms (Yilmaz et al., 2022).

In aquaculture, several species from the *Bacillus* genus are commonly used as probiotics (Boyd et al., 2020; Kuebutornye et al., 2019; Nayak, 2010; Yilmaz et al., 2022). These bacteria are gram-positive and can produce spores. Spores are capable of withstanding a range of adverse conditions such as high temperatures, disinfectants, dry conditions, and low pH, unlike vegetative forms of bacteria (Kuebutornye et al., 2019; Mingmongkolchai & Panbangred, 2018; Nayak, 2010; Wang et al., 2008). Additionally, spores are also bile tolerant, thus they can become established in the intestine, surviving the harsh conditions of the gut transit (Serra et al., 2019).

One of the advantages of using *Bacillus* probiotics is the potential for large-scale production of spores, and as these latent structures are extremely resistant, long-term shelf-storage is also feasible. The conjunction of all these characteristics and the possibility of dehydration of these structures also facilitates their incorporation in feed (Kuebutornye et al., 2019; Serra et al., 2019; Wang et al., 2008).

Fish Isolate 99 (FI99), a *Bacillus subtilis* previously isolated, characterized and identified in ESB by Serra et al. (2019), has been reported as a probiotic with the capacity to hydrolyse NSPs. Its spores have shown high sporulation efficiency, even after being submitted to simulated stomach and gut conditions, thus being able to survive passage through the digestive tract.

Serra et al. (2019) also demonstrated that the inclusion of FI99 spores in PF-challenging aquafeeds (1 x 10^9 spores kg $feed^{-1}$) led to a positive effect on growth, feed efficiency and protein efficiency of ESB juveniles with a tendency to get closer to FM-based diets results, with the inclusion of this probiotic. The values obtained were in between the unsupplemented PF diet (CTR-) and the FM diet (CTR+), showing the need of “fine tuning” the incorporation levels of this probiotic in fish diet.

Due to its carbohydrolytic characteristic, FI99 and its applications have been protected under the patent PCT/IB2019/059131.

2. Objectives

Considering the previous section, the objectives of this work were:

i) Optimize dietary incorporation levels of FI99;

ii) Assess FI99 efficacy against commercial NSPs hydrolyzing enzymes;

iii) Assess the growth and digestible performance of the fish;

iv) Evaluate the potential of FI99 in enhancing fish gut health.

3. Materials and Methods

3.1. Ethical statement

Animal experimentation has been accepted by the Animal Welfare Committee of CIIMAR and was carried out in a registered facility (N16091.UDER), trial ID 181022-dlabrax. All manipulations were carried out by trained personnel in full compliance with national rules following the European Directive 2010/63/EU.

3.2. Spores production and quantification

FI99 is a *B. subtilis*, which is a species included in the Qualified Presumption of Safety (QPS) list of microorganisms intentionally added to feed or food created by the European Food Safety Authority (EFSA), being compliant with the minimum safety requirements to be used as a probiotic (Serra et al., 2019).

Highly purified spores of this bacteria were obtained following the protocol described in Tavares et al. (2013). Briefly, stock isolates of FI99 stored in 30% glycerol at -80ºC were inoculated and grown in a Luria-Bertani (LB) agar medium (BD Difco™) for 48 hours at room temperature to obtain viable colonies. One of the former colonies was selected and incubated in 400 mL of Difco Sporulation Medium (DSM) (BD Difco™), with 400 µL of 1M $Ca(NO_3)_2$, 400 µL of 1M $MnCl_2$, and 400 µL of 1mM $FeSO_4$. This culture was maintained for 48 hours at 37ºC and at 140 rpm to induce sporulation through nutrient exhaustion. The culture was then centrifuged at 20 000g for 10 minutes at 4ºC. The pellet was resuspended in 50 mM Tris-HCl containing 50 µg mL^{-1} of lysozyme and incubated for 1 hour at 37ºC. Afterwards, the pellet was washed with distilled water and then resuspended in 0.05% SDS (sodium dodecyl sulphate) solution. Three more washes with distilled water occurred.

The final pellet was resuspended in 150 µL of distilled water and stored at -20ºC.

Spores were freeze-dried for subsequential incorporation in the experimental diets.

The purity and recovery of the spores were determined through serial dilutions in Bott & Wilson (B&W) salts and plating on LB agar. A 20 minutes' heat treatment at 80ºC was done to eliminate any remaining vegetative cells.

Dietary spores quantification was obtained after resuspending 100 mg of each diet in 1 mL of B&W salts, as described above. Spores quantification was calculated through the counting of visible colonies with the results expressed as Colony-Forming Units (CFUs) per kilogram of feed.

3.3. Experimental diets

Five isoproteic (48%) and isolipidic (18%) diets were formulated. FM inclusion in the diet was limited to 10% in order to formulate challenging diets with high PFs inclusion.

The control diet (CTR) had no spores nor exogenous enzyme inclusion. Incremental inclusion of FI99 were used in Diets 1, 2 and 3 (1×10^9; 7×10^9; 4×10^{10} CFUs kg^{-1}, respectively). An exogenous carbohydrase (Natugrain® TS) was included in the last diet (EXO), to assess FI99 efficacy against a commercial product. Dietary composition and proximate analysis are presented in Table 1.

All ingredients were milled, mixed, and pelleted in the Faculty of Sciences, University of Porto (FCUP), with the usage of a pellet mill (California Pellet Mill, CPM Crawfordsville, IN, EUA). The diets were dried in an oven at 40ºC for 24-48 hours. Diets were stored at room temperature.

Diets for the digestibility trial were formulated similarly to the ones of the growth trial with the only difference being the inclusion of 0.5% Chromium Oxide (Cr_2O_3) as an inert digestibility marker.

Table 1. Experimental diets composition and proximate analysis

	Diets				
	CTR	**Diet 1**	**Diet 2**	**Diet 3**	**EXO**
Ingredients % dry weight basis					
LT Fishmeal[1]	10.0	10.0	10.0	10.0	10.0
Soluble fish protein concentrate[2]	5.0	5.0	5.0	5.0	5.0
Rapeseed meal[3]	12.0	12.0	12.0	12.0	12.0
Wheat gluten[4]	10.3	10.3	10.3	10.3	10.3
Whole wheat[5]	0.7	0.7	0.7	0.7	0.7
Soybean meal [6]	20.0	20.0	20.0	20.0	20.0
Soy Protein Concentrate[7]	10.0	10.0	10.0	10.0	10.0
Sunflower meal[8]	12.0	12.0	12.0	12.0	12.0
Fish Oil	15.6	15.6	15.6	15.6	15.6
Vitamin premix[9]	1.0	1.0	1.0	1.0	1.0
Mineral premix[10]	1.0	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5
Binder[11]	1.0	1.0	1.0	1.0	1.0
Taurine[12]	0.3	0.3	0.3	0.3	0.3
Dibasic calcium phosphate	0.5	0.5	0.5	0.5	0.5
FI99 CFU kg^{-1}	-	1×10^9	7×10^9	4×10^{10}	-
Enzymatic Cocktail[13]	-	-	-	-	0.04
Proximate analysis (% DM)					
Dry matter	87.7	88.6	89.5	90.8	86.6
Crude Protein	48.4	47.6	47.2	46.9	48.2
Crude Lipid	19.0	18.5	18.2	17.7	18.5
Ash	8.8	6.6	6.2	7.5	6.3
Gross energy (kJ/g)	24.3	24.8	24.8	24.0	25.1

CP = crude protein; DM = dry matter; GL = gross lipid.

[1]Steam dried LT-FM, Pesquera Centinela, Chile, (CP: 86.5% DM; GL: 9.2% DM).

[2]Sorgal, S.A. Ovar, Portugal (CP: 77.8% DM; GL: 8.2% DM).

[3] Sorgal, S.A. Ovar, Portugal (CP: 44.3% DM; GL: 1.1% DM).

[4] Sorgal, S.A. Ovar, Portugal (CP: 78.3% DM; GL: 3.5% DM).

[5] Sorgal, S.A. Ovar, Portugal (CP: 15.6% DM; GL: 1.5% DM).

[6] Sorgal, S.A. Ovar, Portugal (CP: 50.0% DM; GL: 0.9% DM).

[7] Sorgal, S.A. Ovar, Portugal (CP: 67.2% DM; GL: 0.2% DM).

[8] Sorgal, S.A. Ovar, Portugal (CP: 30.4% DM; GL: 0.4% DM).

[9]Vitamins (mg/kg diet): retinol, 18,000 (IU/kg diet); cholecalciferol, 2,000 (IU/kg diet); α-tocopherol, 35; menadione sodium bisulphate, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[10]Minerals (mg/kg diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g/kg diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.44 (g/kg diet).

[11]Aquacube. Agil, UK.

[12]Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.

[13] Natugrain®, BASF SE, Lampertheim, Germany: endo-1, 4-β-xylanase, 5600 (TXU/g); endo-1,4-β-glucanase 2500 (TGU/g).

3.4. Growth trial

The growth trial was conducted in the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR).

The fish were provided by Maresa S.A. (Ayamonte, Huelva, Spain), and after transportation stayed in quarantine for 15 days at CIIMAR being fed with a commercial diet containing 46% crude protein and 16% crude fat (dry matter basis) from Pelagia™ Bradbenken, Bergen, Norway.

The experimental system consisted of a thermoregulated Recirculatory Aquaculture system (RAS) equipped with 15 fiberglass tanks, each with a capacity of 250 L.

During the trial, the temperature averaged 21.4 ± 0.5ºC, salinity 30.0 ± 1.9 g L^{-1}, dissolved oxygen 8.5 ± 0.3 mg L^{-1}, and nitrogenous concentration was kept under 0.02 mg L^{-1}. The photoperiod was regulated to ensure 12 h light/ 12 h dark.

Fifteen groups of 25 fish with an initial mean body weight of 9.8 ± 0.04 g were randomly allotted to the tanks, and diets were tested in triplicate. Feeding consisted of two time periods (5 hours' intervals from the first feeding) until apparent visual satiety. Fish were fed 6 days a week. Maximum care was taken to avoid pellet waste and to ensure that all diet was consumed by the fish. The trial lasted 71 days.

3.5. Digestibility trial

The digestibility trial was conducted in the same institution - CIIMAR. The trial occurred in a thermo-regulated recirculating water system equipped with 12 fiberglass tanks with

60 L water capacity, and feces settling columns paired to the outlet of each tank, designed according to Cho (1990).

Twelve fish, from the growth trial, with mean body weight of 36.6 ± 0.2 g were randomly assigned to each tank and the experimental diets were tested in duplicate. The fish were fed until apparent visual satiation. The trial lasted 15 days.

During the trial, salinity averaged 30.4 ± 2.4 g L^{-1}, temperature 21.4 ± 0.3ºC and dissolved oxygen 8.2 ± 0.1 mg L^{-1} and nitrogenous concentration was kept under 0.02 mg L^{-1}. The fish were fed twice a day (5 hours' interval), 7 days a week. Collection of the feces was done in the morning before any feeding. The system was daily cleaned after the afternoon feeding.

Feces were centrifuged (3000 g, 10 minutes) and stored at -20ºC until later analysis.

Prior to conducting proximal analysis, the fecal samples were subjected to a drying process at ± 40°C for a day, after which they were ground and stored at room temperature until the analysis performance.

Apparent digestibility coefficients (ADC) of diets components (dry matter, protein, lipids, and energy) were calculated as follows:

$$\mathbf{ADCdiet} = \left(\mathbf{1} - \frac{\mathbf{dietary\ Cr2O3\ level} \times \mathbf{feces\ nutrient\ or\ energy\ level}}{\mathbf{feces\ Cr2O3\ level\ x\ dietary\ nutrient\ or\ energy\ level}}\right) \times \mathbf{100}$$

3.6. Sampling

Fish in each tank were feed-deprived and bulk weighted at the beginning and at the end of the growth trial, being previously anesthetized with 0.3 mL L^{-1} ethylene glycol monophenyl ether. Sampling occurred 5 hours after the morning feeding to ensure that feed was present in the gut for further analysis. A total of 6 fish per tank (18 per experimental group) were sacrificed by decapitation after anesthetization.

Histomorphological samples of 3 fish intestines per tank (9 per experimental group) were obtained accordingly to Magalhães et al. (2020). After the removal of the adjacent adipose and connective tissue of the intestine, a small portion of the distal intestine (DI) was collected. This portion was selected with use of its characteristics: higher diameter, and darker tone than the mid intestine, as well as the presence of the annular ring, and

due to its higher sensibility to dietary treatments. The DI portion was cleaned in phosphate-buffered saline (PBS) and fixed in phosphate-buffered formalin (4%, pH 7.4) for 24 hours, and then transferred into ethanol 70%. The remaining intestine was flash-frozen in dry ice and stored at -80ºC for later analysis of oxidative stress enzymes.

The entire intestine of the remaining 3 fish was collected, flash-frozen in dry-ice, and stored at -80ºC for later digestive enzymatic activity measurements.

3.7. Bromatological Analysis

Dry matter, protein, lipids and ash contents of the ingredients and diets were analyzed according to Association of Official Analytical Chemist methods (AOAC, 2000). Feces protein and energy were assessed according to the same methods.

Briefly, dry matter was assessed through drying the samples at 105ºC in an oven until constant weight. Incineration at 405ºC for 12 hours allowed to obtain the non-organic (ash) portion of the samples. Protein content (with assumption N x 6.25) was obtained according to the Kjedahl method, followed by acid digestion on Kjeltec digestor and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively). Lipids through extraction in an organic solvent (petroleum ether) using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046).

Diet and feces energy content were obtained by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261).

The chromic oxide in the diets and feces were determined through acid digestion following Furukawa & Tsukahara (1966) methodology.

3.8. Digestive enzyme activity

Whole gut was used to assess the activity of total alkaline protease (TAP), trypsin, α-amylase, and lipase. Guts were homogenized in a cold buffer (1:5 w/v) of 50 mM Tris-HCl buffer (pH 7.5). The homogenates were centrifuged at 30 000 g for 15 minutes at 4ºC. The supernatant was aliquoted and stored at -80ºC until digestive enzymes analysis. Enzymatic activities were assessed according to Couto et al. (2016).

Briefly, TAP was measured by the casein-hydrolysis method. The reaction mixture contained casein (1% w/v; 0.125 mL), a buffer solution (0.1 M Tris-HCl, pH 9; 0.125 mL), and the previously mentioned homogenate supernatant (0.05 mL). This mixture was incubated at 37ºC for 1 hour.

The reaction was stopped with the addition of 0.3 mL trichloroacetic acid solution (TCA) at 8% w/v. Samples rested for 1 hour at 4ºC and then were centrifuged at 1 800 g for 10 minutes. Supernatant absorbance was measured at 280 nm. Control blanks for each sample were prepared adding the supernatants from the homogenates after the incubation period.

For trypsin activity, Nα- benzoyl-L-arginine 4-nitroanilide hydrochloride (1 mM BAPNA) was used as the substrate. The trypsin buffer was composed of 50 mM Trizma ® base and 20 Mm $CaCl_2$, pH 8.2. The production of p-nitroanaline (molar extinction coefficient, 8.8 mM^{-1} cm^{-1}) was monitored at 37ºC and recorded at 410 nm.

Using the formation rate of 2-chloro-4-nitrophenol at 37ºC, α-amylase activity was assessed. The formation of 2-chloro-4-nitrophenol was measured at 405 nm. A commercial kit from Spinreact, Girona, Spain (ref. 41201) was used for this effect.

A kit from the same company (Spinreact, ref. 1001274) was utilized to assess lipase activity. For this purpose, the formation rate of methylresorufin (with molar extinction coefficient, 60.65 mM^{-1} cm^{-1}) was assessed photometrically at 580 nm at 37ºC. This rate is by convention proportional to the concentration of catalytic lipase in the samples.

All the enzyme activities were expressed as miliunits per mg of hepatic soluble protein (specific activity). The protein concentration were determined utilizing bovine serum albumin as standard as described in Bradford (1976).

The amount of enzyme required to catalyze the hydrolysis of 1µmol of substrate per min under the assay conditions was considered as one unit of the enzyme.

All these measurements were performed in a Multiskan Go microplate reader (model 5111 920; Thermo Scientific, Nanjin, China).

3.9. Evaluation of fish oxidative status

Oxidative status of the fish was assessed in the gut. For that purpose, the gut (1:5 w/v) was homogenized in a cold solution of phosphate potassium buffer (0.1 M, pH 7).

Thereafter, the homogenates were centrifuged at 13 400 g for 25 minutes at 4ºC, and the supernatants were aliquoted and stored at -80ºC for later analysis.

Lipid peroxidation (LPO) in the gut was evaluated using malondialdehyde (MDA) according to Magalhães et al. (2020). This marker in the presence of thiobarbituric acid generates thiobarbituric acid reacting substances (TBARS) that have specific colourations. These colouration variations were measured by spectrophotometry at 535 nm. The results were obtained with the use of an MDA calibration curve.

Glutathione reductase (GR) was determined through oxidation of NADPH, associated with the reduction of oxidized glutathione (GSSG) (Carvalhais et al., 2021).

Glutathione peroxidase (GPX) activity was measured by the NADPH consumption rate (NADPH to $NADP^+$) by GSSG produced by GPX and reduced by glutathione reductase (GR) following the steps of Carvalhais et al. (2021).

Superoxide dismutase (SOD) activity was measured with the use of xanthine/xanthine oxidase as the source of superoxide radicals. The amount of enzyme necessary to produce 50% inhibition of the ferricytochrome reduction rate was considered as one unit of activity. An enzymatic kit (RANSOD™, Randox) was used for this effect.

The catalase (CAT) activity was assessed with the decrease in hydrogen peroxide concentration as described in Aebi (1984).

All the enzyme activities were expressed as miliunits per mg of hepatic soluble protein (specific activity) except for CAT and SOD which were expressed as units per mg protein. Soluble protein concentration was obtained using a Sigma-Aldrich Protein assay kit and bovine serum albumin as standard according to Bradford (1976).

The amount of enzyme required to catalyze the hydrolysis of 1µmol of substrate per min under the assay conditions was considered as one unit of the enzyme.

All oxidative stress enzymes measurements were performed in a Multiskan Go microplate reader (model 5111 920; Thermo Scientific, Nanjin, China).

3.10. Histological analysis

The DI was the focus of histological analysis. The histological samples were obtained, processed, and sectioned using standard histological technics. The samples were fixed

with phosphate-buffered formalin (10%) and stored in alcohol at 70% until analysis. After processing, sections were blemished with haematoxylin and eosin (H&E stain).

A blind evaluation of the preparations was realized with a particular focus on any inflammatory changes, considering the criteria present in Krogdahl et al. (2003): i) widening and/or shortening of intestinal folds; ii) loss of supranuclear vacuolization in enterocytes present in the intestinal epithelium; iii) widening of the lamina propria of the intestinal folds; iv) infiltration of immunological cells (leucocytes) in the lamina propria and submucosa.

Regarding the evaluation method, a continuous scale scoring system ranging from 1 (without morphological alterations) to 5 (extreme morphological alterations) was used to calculate the overall average alterations of the previously mentioned parameters.

3.11. Statistical analysis

Statistical analysis of the results was performed in the SPSS software package for Mac (IBM® SPSS® Statistics, version 27, release 27.0.1.0). Homogeneity and normality were assessed for all data (Levene and Shapiro-Wilk tests, respectively). Results were analysed by one-way analysis of variance (ANOVA). Significant differences between means were evaluated by Tuckey's multiple range test. The probability level for rejection of the null hypothesis was $p < 0.05$.

Specific activity of digestive enzymes was log-transformed to meet ANOVA requirements (homogeneity and normality).

Histological data was analysed by the Kruskal-Wallis test as data was neither normal nor homogeneous and cannot be normalized.

4. Results

4.1. Spores quantification

Spores quantification in growth diets displayed concentrations of: D1: 6.5 x 10^8 CFU kg^{-1}; D2: 2.5 x 10^9 CFU kg^{-1}; D3: 9.6 x 10^9 CFU kg^{-1} (Figure 2) Growths' CTR and EXO diets did not display any spores.

The digestibility diets exhibited the presence of FI99 spores in both the CTR and EXO diets. Spores quantification in digestibility diets displayed concentrations of: CTR: 1.0 x 10^8 CFU kg^{-1}; D1: 3.2 x 10^8 CFU kg^{-1}; D2: 1.3 x 10^9 CFU kg^{-1}; D3: 3.7 x 10^9 CFU kg^{-1}; EXO: 7.3 x 10^7 CFU kg^{-1} (Figure 2).

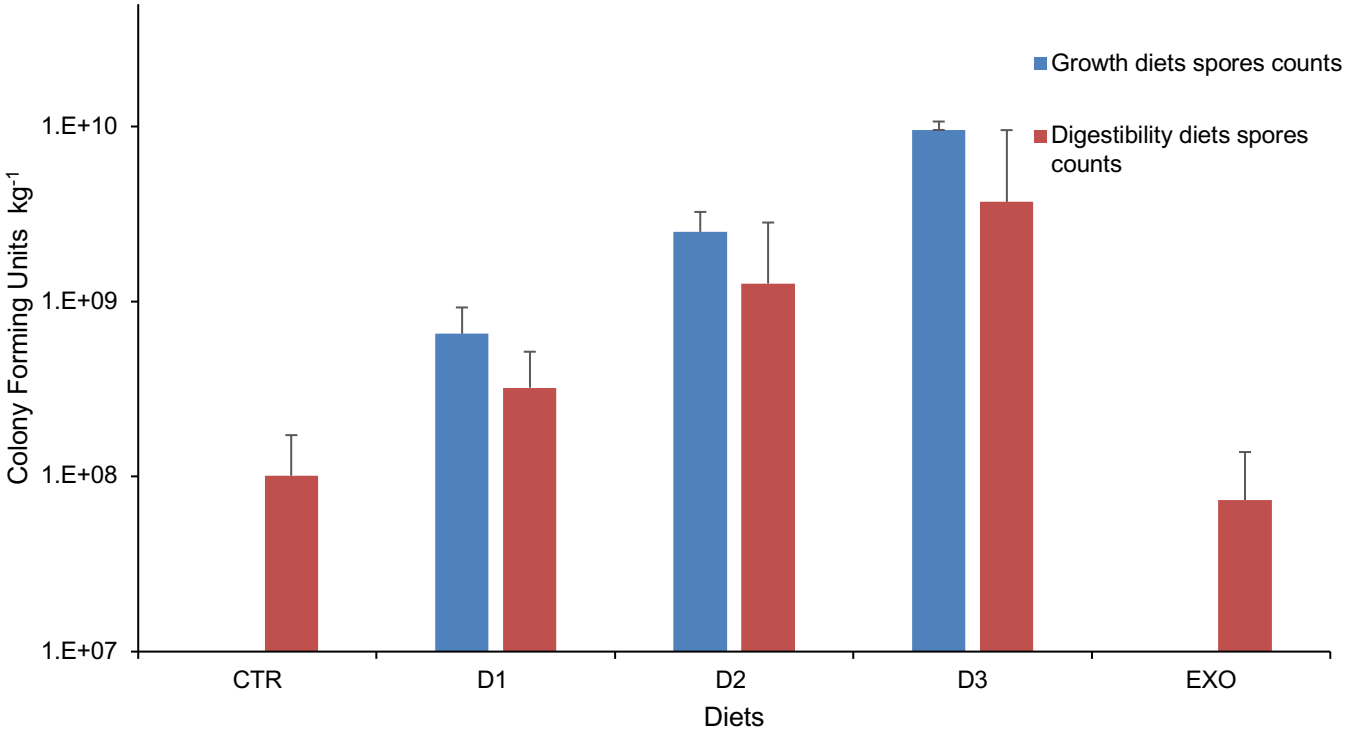

Figure 2. Fish Isolate 99 (FI99) spores quantification in growth and digestibility diets. Data are presented as mean (n=3). Error bars represent standard deviation.

4.2. Growth trial

Fish hastily accepted the experimental diets and mortality during the trial was lower than 3%. Mortality was only observed in diets D1, D2 and D3 but was not different from the CTR diet.

Inclusions of FI99 had no significant effect on final body weight, weight gain, daily growth index, feed intake, feed efficiency, protein efficiency ratio, and protein, lipid and energy intake (Table 2). EXO diet also had no significant effects on the already mentioned parameters.

Table 2. Growth performance and feed utilization efficiency of European sea bass fed with the experimental diets

	Diets						
	CTR	**D1**	**D2**	**D3**	**EXO**	**SEM**	**ANOVA**
Initial body weight (g)	9.8	9.8	9.8	9.8	9.8	0.0	0.35
Final body weight (g)	35.4	34.1	34.3	33.0	35.0	1.7	0.31
Weight gain (% initial weight)	261.1	248.9	249.1	236.6	257.6	17.0	0.86
Weight gain (g kg ABW^{-1*} day^{-1})	3.7	3.6	3.6	3.4	3.7	0.2	0.86
Daily growth index[1]	1.6	1.6	1.6	1.5	1.6	0.1	0.86
Feed intake	26.3	25.8	25.9	26.2	26.0	0.4	0.92
Feed efficiency[2]	0.6	0.6	0.6	0.6	0.6	0.0	0.24
Protein efficiency ratio[3]	1.3	1.3	1.3	1.3	1.3	0.0	0.75
N intake (g kg ABW^{-1*} day^{-1})	2.0	2.0	2.0	2.0	2.0	0.0	0.53
Lipid intake (g kg ABW* day^{-1})	5.0	4.8	4.7	4.6	4.8	0.1	0.09
Energy intake (g kg ABW* day^{-1})	638.8	641.5	641.4	627.7	652.8	11.0	0.63
Survival (%)	100.0	97.4	98.7	98.7	100.0	1.0	0.39

Mean values and pooled standard error of the mean (SEM) are presented for each parameter (n = 3). P-value displayed under ANOVA. Lack of superscript letters indicate lack of significant difference between dietary treatments (P > 0.05).

1 DGI: ((final body weight1/3 − initial body weight1/3)/time in days) × 100.

2 FE: (wet weight gain/dry feed intake).

3 PER: (wet weight gain/crude protein intake).

* Average body weight: (initial body weight + final body weight)/2.

4.3. Digestibility trial

The fish readily accepted experimental diets and no mortality was observed during trial.

Apparent digestibility coefficient of dry matter, protein and energy of the experimental diets are present in Table 3.

Table 3. Apparent digestibility coefficient (ADC) of the experimental diets

	Diets						
	CTR	**D1**	**D2**	**D3**	**EXO**	**SEM**	**ANOVA**
ADC (%)							
Dry matter	64.1 ^{ab}	58.2 ^{a}	61.4 ^{ab}	57.2 ^{a}	67.8 ^{b}	1.9	0.01
Protein	93.8 ^{ab}	92.7 ^{a}	92.7 ^{a}	92.9 ^{a}	94.8 ^{b}	0.4	0.02
Energy	80.2 ^{b}	77.3 ^{a}	78.3 ^{a}	76.9 ^{a}	82.3 ^{c}	1.0	<.001

Mean values and pooled standard error of the mean (SEM) are presented for each parameter (n = 2). Superscript letters indicate significant difference between dietary treatments. P-value displayed under ANOVA.

Apparent digestibility of dry matter was highest (67.8%) with exoenzyme supplementation (EXO diet), albeit it was not statistical different from the CTR and D2 diets (64.1% and 61.4%, respectively). Notably, the EXO diet's ADC of dry matter was significantly different from both the D1 and D3 diets (58.2% and 57.2%, respectively). On the other hand, CTR dry matter ADC was not significantly different from the FI99 or exoenzyme supplemented diets.

Exoenzyme supplementation led to an enhancement in protein ADC when compared to the FI99-supplemented diets. Exoenzyme supplementation increased protein ADC by 2.1%, 2.1% and 1.9%, in comparison to D1, D2 and D3 diets, respectively. No significant statistical differences were observed in protein ADC between CTR, EXO, and the FI99-supplemented diets.

Exoenzyme supplementation led to a significant increase in energy ADC (82.3%) comparing to CTR diet (80.2%). FI99 incorporation in the three crescent levels significantly diminished energy ADC in diets D1, D2, and D3 by 2.9%, 1.9% and 3.3%, respectively comparing to CTR diet. Energy ADC was not different between diets D1, D2 and D3.

4.4. Digestive enzyme activity

Despite CTR and D3 displaying highest TAP, trypsin and lipase activities than the other experimental groups, no significant differences were observed in digestive enzyme activity (Table 4).

Table 4. Specific activities (mU mg protein^{-1}) of Total Alkaline Proteases, Trypsin, Lipase and α-Amylase in European sea bass whole-gut fed with the experimental diets

	Diets						
	CTR	**D1**	**D2**	**D3**	**EXO**	**SEM**	**ANOVA**
Total Alkaline Proteases	1 170.7	1 007.9	913.6	1 179.6	1 050.1	113.8	0.33
Trypsin	324.7	229.3	240.6	325.5	263.5	43.9	0.31
Lipase	18.4	14.5	14.0	20.8	16.6	2.1	0.13
α-Amylase	82.0	63.0	62.8	80.5	82.5	9.2	0.24

Mean values and pooled standard error of the mean (SEM) are presented for each parameter (n = 9). P-value displayed under ANOVA. Lack of superscript letters indicate lack of significant difference between dietary treatments (P > 0.05).

4.5. Oxidative stress enzyme activity

Enzymatic activities of the different oxidative stress indicators are present in Table 5.

No significant statistical differences were found in the antioxidant activity of CAT, GPX and GR in the gut of European sea bass, between experimental diets. No statistical differences were also observed in LPO. Relating to SOD activity, diet D2 did not display any statistical differences between any dietary treatments. SOD activity of fish fed with EXO diet was higher than CTR and D3 diets. SOD activity of FI99- supplemented diets were not different from the CTR diet.

Table 5. Intestine antioxidant enzyme activity of European sea bass fed with the experimental diets

	Diets						
	CTR	**D1**	**D2**	**D3**	**EXO**	**SEM**	**ANOVA**
CAT	17.8	13.0	13.5	11.5	16.1	1.63	0.07
GPX	5.1	6.3	4.9	4.9	5.4	0.56	0.38
GR	3.3	2.9	3.0	2.9	3.2	0.17	0.31
SOD	59.3 ^{ab}	104.9 ^{bc}	86.3 ^{abc}	48.8 ^{a}	113.0 ^{c}	11.9	0.001
LPO	11.6	11.2	16.3	12.1	10.5	2.28	0.408

Mean values and pooled standard error of the mean (SEM) are presented for each parameter (n = 9). P-value displayed under ANOVA. CAT activity U mg protein^{-1}, GPX mU mg protein^{-1}, GR as mU mg protein^{-1}, SOD as U mg protein^{-1}, and LPO as nmol of TBARS formed mg^{-1} tissue.

4.6. Histology

DI histological examination using the scoring system presented in the materials and methods section revealed no significant differences among experimental diets. The key aspects assessed included the dimensions of mucosal folds (height), width, cellularity, and leukocyte infiltration within the lamina propria and submucosa, as well as the alignment and vacuolization of enterocytes (Table 6, Figure 3).

Table 6. Histological score-based evaluation of European sea bass distal-intestine fed with the experimental diets

	Diets					
	CTR	**D1**	**D2**	**D3**	**EXO**	**SEM**
Fold height	1.0	1.0	1.0	1.0	1.0	0.00
Lamina propria width	1.0	1.0	1.0	1.0	1.0	0.00
Lamina propria cellullarity	1.0	1.0	1.0	1.0	1.0	0.00
Submucosa width	1.0	1.0	1.0	1.0	1.1	0.05
Submucosa cellularity	1.0	1.0	1.0	1.0	1.0	0.00
Eosinophilic granulocyte cells	1.1	1.3	1.0	1.0	1.0	0.09
Enterocytes allignment	1.0	1.0	1.0	1.0	1.0	0.00
Enterocytes vacuolization	1.3	1.0	1.1	1.0	1.1	0.10
Mean score	1.05	1.04	1.01	1.0	1.02	0.02

Mean values and pooled standard error of the mean (SEM) are presented for each parameter (n = 9). Lack of superscript letters indicate lack of significant difference between dietary treatments ($P > 0.05$).

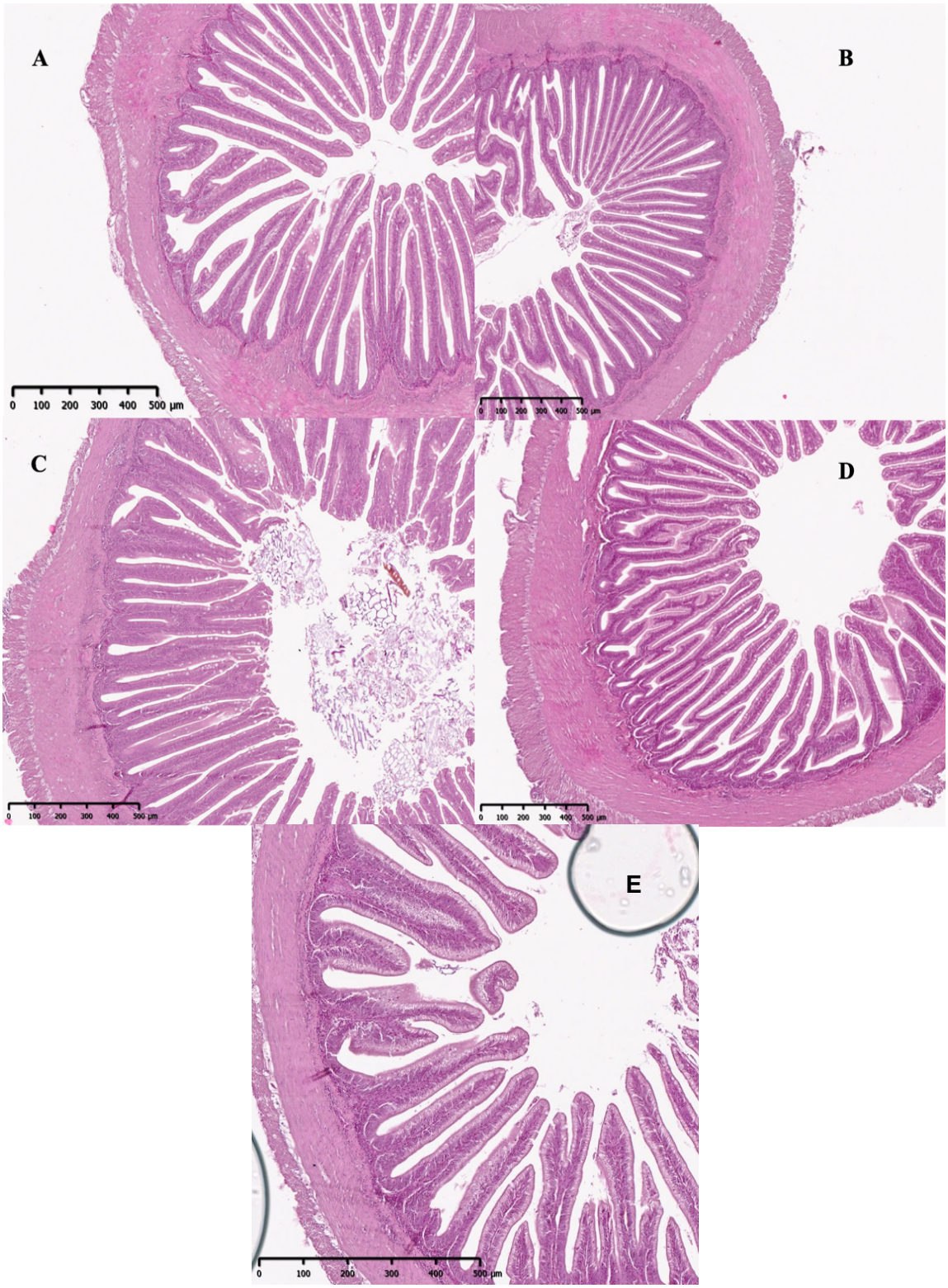

Figure 3. Histological preparations of distal intestine of European sea bass fed with experimental diets (A-CTR; B-D1; C-D2; D-D3; E-EXO). Pictures recorded with NDP.view2 (free edition version 2.929). Normal morphology without inflammation signs. H&E stain.

5. Discussion

The research and utilization of probiotics in aquaculture have experienced significant growth since their introduction in the late 1990s (Chuphal et al., 2021; Senok et al., 2005). Studies generally show that probiotics inclusion in aquafeeds may lead to positive effects not only in the growth and feed utilization (Adorian et al., 2019; Olmos et al., 2022; Rohani et al., 2022), but also in the immune status of fish (Rohani et al., 2022; Zaineldin et al., 2018). Among the different probiotics used, *Bacillus* spp. showed several advantages such as their fast growth rate, ability to produce a different range of digestible enzymes, and most importantly, their ability to produce spores, thus facilitating their incorporation in aquafeeds (Boyd et al., 2020; Kuebutornye et al., 2019; Nayak, 2021).

In general, *Bacillus* spp. supplementation in aquafeeds can promote fish growth performance. For instance, Tachibana et al. (2021) reported that 0.04% and 0.08% dietary incorporation of a probiotic cocktail (*Bacillus subtilis*: 1.6 x 10^{10} CFU g^{-1} feed and *Bacillus licheniformis*: 1.6 x 10^{10} CFU g^{-1} feed) in plant based diets, improved growth performance of Nile tilapia (*Oreochromis niloticus*). Similar results were described in olive flounder (*Paralichthys olivaceus*), where the dietary incorporation of *Bacillus subtilis* (5×10^7 CFU g^{-1} feed) significantly enhanced the growth performance of the fish (Cha et al., 2013).

Despite this, some studies also showed that the inclusion of *Bacillus* spp. may not always affect fish performance. Addo et al. (2017) demonstrated that the inclusion of different *Bacillus* strains at a concentration of 4 x 10^7 CFU g^{-1} feed in plant-based diets (FM inclusion of 4%) did not promote growth in Nile tilapia. Moreover, Chouayekh et al. (2023) reported that *Bacillus amyloliquefaciens* (1x 10^7 CFU g^{-1} feed) had no effect on the growth performance and parameters such as weight gain and feed conversion ratio of European sea bass.

In the present study, increasing dietary incorporation levels of *Bacillus subtilis* FI99 did not produce any significant effect on the growth performance of European sea bass, thus in line with the results obtained by Addo et al. (2017) and Chouayekh et al. (2023). Nevertheless, the spores' incorporation in our study were significantly lower than the ones in the two previously mentioned studies. Additionally, diet EXO, containing endo-1, 4-β-xylanase (5600 TXU g^{-1}) and endo -1, 4-β-glucanase (2500 TGU g^{-1}), did not present growth and feed utilization differences between the control group and the probiotic groups.

These results can possibly be explained by the lack of significant differences between probiotic or exogenous enzyme-supplemented diets in dry matter and protein ADCs, compared to the control diet. This, in turn, could be explained by the lack of significant differences in the measured digestive enzyme activities between experimental diets and the control diet.

It is important to note that both CTR and EXO digestibility diets displayed FI99 spore's contaminations (1 x 10^8 CFU kg^{-1} and 7.3 x 10^7 CFU kg^{-1}, respectively). These contaminations were most likely due to the inefficient/inappropriate cleaning of the pelletizer (deep cleaning with ethanol 70%), as these diets were manufactured at a later time than the growth diets.

These contaminations may have affected digestibility results, and in future growth and digestibility diets ought to be manufactured *in tandem*. Furthermore, the utilization of stronger disinfectants is required for the cleaning all the equipment's utilized in the manufacturing of experimental diets.

ADC of dry matter displayed results comparatively low (57.2-67.8%), contrasting with other studies involving high dietary PF incorporations in ESB diets, as exemplified by Magalhães et al. (2018) (69.9 - 88.4%) with diets containing 64% PF inclusions. These results, however, are not aligned with the expected outcomes as both FI99 and Natugrain® are known for their carbohydrolytic potential, as evidenced by previous research (Fernandes et al., 2021; Magalhães et al., 2018; Serra et al., 2019).

FI99 is described by Serra et al. (2019), as having genes coding for the production of both glucanases (β-glucanase (*bglS*)) and xylanases (arabinoxylan arabinofuranohydrolase (*xynD*)) and for the metabolization of different cellulose and hemicellulose monomers (glucose and xylose, respectively). As such we could expect a higher utilization of dietary fibers, leading to a higher dry matter ADC compared to the control diet. Authors also reported that the inclusion of probiotics can modify digestive enzyme activities, as exemplified by Liu et al. (2017). Thus, Nile tilapia fed a commercial diet supplemented with *Bacillus subtilis* HAINUP40 (1 x 10^8 CFU g^{-1} feed) displayed higher protease and amylase activities after 4 and 8 weeks compared to the unsupplemented diet. In contrast to this, our probiotic did not display differences in the main digestive enzyme activities.

Considering this, FI99 spore's supplementation, in three different incorporation levels, had no effect, on dry matter and protein ADC compared to the control diet. FI99 in all

three supplementation levels did however significantly diminish energy ADC. No differences were also observed between FI99 supplemented diets (D1, D2 and D3) in dry matter, protein, and energy ADCs.

Natugrain® is an enzymatic cocktail composed of endo-1,4-β-xylanase, and endo-1,4-β-glucanase. Endo-glucanases are enzymes whose main role is to hydrolyze internal bonds of the cellulose chain (Horn et al., 2012), whereas endo-xylanases are responsible for the degradation of xylan, the main constituent of hemicellulose (Collins et al., 2005). Studies showed that dietary enzyme supplementation may be able to influence endogenous proteolytic, lipolytic, and amylolytic enzyme activities in certain fish species. Thus, Mori (*Cirrhinus mrigala*) fed with plant-based diets had increased protease, amylase and lipase activities by dietary xylanase inclusion (1882 U kg^{-1}) (Nadeem et al., 2022). Similar results were described in Jian carp (*Cyprinus carpio* var. Jian) as trypsin, chymotrypsin, lipase and amylase activities were enhanced when fish were fed plant-based diets supplemented with xylanase (up to 1070-1480 U kg^{-1} diet) (Jiang et al., 2014). Magalhães et al. (2016) demonstrated that white seabream (*Diplodus sargus*) fed a plant-based diet (68% PF) supplemented with NSPases displayed higher amylase and lipase activities compared to the unsupplemented diet. Despite this, Magalhães et al. (2018) studied the potential of Natugrain® as a dietary carbohydrolytic exoenzyme supplementation (0.02 and 0.04% dry weight basis) in plant-based diets in ESB (64% PF incorporation). Here, the supplementation of Natugrain® at a 0.04% did not induce any modulations in intestinal enzyme activities, mirroring the results of our study.

The EXO diet did not yield significant improvements in dry matter and protein ADCs when compared to the CTR diet, contrary to the findings reported by Magalhães et al. (2018). However, it is worth noting that energy ADC exhibited a significant enhancement upon the inclusion of this enzymatic cocktail. This is in line with the data reported by the same authors and suggests that the increased energy ADC may be attributed to a more effective utilization of dietary fibers. Furthermore Magalhães et al. (2018) concluded that the better utilization of dietary fibers might be due to the hydrolysis of NSPs, as the breaking of these polysaccharides may reduce digesta viscosity, enhancing the mixing of endogenous and exogenous digestive enzymes with the nutrients.

Overall, the enzymatic cocktail displayed higher dry matter, protein and energy ADC values compared to the probiotic-supplemented diets. Indeed, dry matter ADC of the EXO diet was significantly higher than D1 and D3 diets values. This effect was also reflected in energy ADC and could be possibly explained due to a higher digestibility of

dietary fibers. With the present results, we can hypothesize that Natugrain® enzymes were able to hydrolyse the dietary fibers to a higher extent, compared to FI99, or that FI99 was not able to produce the required concentration of enzymes to help the host to breakdown the dietary fibers.

Therefore, it is essential to conduct an analysis of the ADC of dietary fibers as it can provide valuable insights into their different components, including acid detergent fibers (ADF), neutral detergent fiber (NDF), and lignin. Moreover, it is necessary to assess the activity of specific carbohydrolytic enzymes for NSPs such as β-xylanase and β-glucanase.

The lack of significant differences in digestive enzyme activity and ADC values with the probiotic supplemented diets could also possibly be explained by the non-colonization of the fish gut by FI99. In this sense, microbiota analysis of the fish gut could provide an insight on ESB gut microbiota modulation and also demonstrate if FI99 was able to colonize the gastrointestinal apparatus of the fish.

The intestinal mucosa is fundamental for the nutrients absorption, as such, critical for the proper function of the gut (Zhang et al., 2018). In salmonids, the inclusion of soybean products, due to high concentration of dietary fibers, can lead to alterations of gut morphology, such as shortening of the mucosal folds and gut inflammation with infiltration of lymphocytes, macrophytes and granular cells, leading to a reduction of macromolecules absorption and increased permeability (Krogdahl et al., 2010). This inflammatory process has been described in salmonids such as Atlantic salmon (Baeverfjord & Krogdahl, 1996) and Rainbow trout, but also in non-salmonid species such as Seabream (Rimoldi et al., 2016).

Rimoldi et al. (2016) reported that ESB fed with a diet with 30% soy protein (16.7% soybean meal and 12.8% as full-fat soy) showed signs of gut inflammation, with high mobilization of inflammatory cells. Despite this, Kotzamanis et al. (2020) also demonstrated that ESB can be fed with high dietary concentrations of soybean meal and soy protein concentrate (32%) without causing major histomorphologic changes in the distal gut of the fish, with only mild enteritis (infiltration of white blood cells) present in some individuals of all experimental groups.

Furthermore, a previous study by Bonvini et al. (2018) demonstrated that ESB gut histomorphology is not affected in fish fed high PF incorporations (30% soybean meal inclusions). In the current research, we noted that diets rich in PF, composed of 30% dry

weight soy protein (20% soybean meal and 10% soy protein concentrate), exhibited no discernible impacts on the histomorphology of the gut. Furthermore, there were no noticeable signs of an inflammatory process in this context. Additionally, we found that the inclusion of either FI99 spores, up to 9.6 x 10^9 CFU kg^{-1} feed, or Natugrain® (0.04 % dry weight basis) have no negative visible effect in the histomorphological structural architecture of the distal gut. These results go in accordance with literature as generally authors do not report negative effects in diets with probiotics or exogenous enzymes inclusions.

The absence of positive effects resulting from either FI99 supplementation or enzyme use could potentially be attributed to the diet's inability to induce an inflammatory process within the gastrointestinal tract.

During metabolic processes, all aerobic organisms produce byproducts such as reactive oxygen species (ROS) (Birnie-Gauvin et al., 2017). ROS can be superoxide, hydrogen peroxide and hydroxyl radical and these can be formed endogenously from the mitochondrial electron transport chain, lysosome activity, among others (Birnie-Gauvin et al., 2017; Chowdhury & Saikia, 2020; Hoseinifar et al., 2021). These by-products of the metabolism are essential for the organism, in certain functions such as the hosts' defense system and cellular signaling, but in excess they can be highly reactive, and in turn, have negative effects in lipid and protein structure, and in nucleic acids, damaging the DNA (Birnie-Gauvin et al., 2017; Chowdhury & Saikia, 2020; Hoseinifar et al., 2021; Vinagre et al., 2012).

To prevent high accumulations of ROS and minimize its damages, fish as well as other organisms, have enzymatic antioxidant defense systems, such as superoxide dismutase (capable of catabolizing superoxide forming oxygen and oxygen peroxide), catalase (responsible for partial erasure of hydrogen peroxide with the formation of oxygen and water), and glutathione peroxidase (with the function of eliminating hydrogen peroxide when glutathione is present, forming oxidized glutathione and water). The oxidized glutathione is later reduced by glutathione reductase to form active glutathione (Chowdhury & Saikia, 2020; Hoseinifar et al., 2021). When ROS formation and concentration are excessively higher than the action of this antioxidant system, the organism enters in oxidative stress (Chowdhury & Saikia, 2020; Hoseinifar et al., 2021). Lipid peroxidation is a chain reaction where polyunsaturated fatty acids are damaged by oxidative stress (Chowdhury & Saikia, 2020), and due to its relevance, its products (malondialdehyde, MDA) are used as biomarkers of lipid damage, thus providing an

insight into overall oxidative stress levels (Birnie-Gauvin et al., 2017; El-Sayed & Izquierdo, 2022).

Diet can be a stress-inductor, contributing to oxidative stress (Fernandes et al., 2022). Although NSPs can sometimes trigger the activation of phagocytes and facilitate an elevation in ROS levels, thereby enhancing the fish's immune system, they can also potentially jeopardize the cellular integrity and the organism health in acute situations (Enes et al., 2012). Considering this, the inclusion of probiotics and exogenous enzymes may modulate the enzymatic antioxidant capacity of fish. Wang et al. (2019) described that the enzymatic antioxidant activity was positively affected in Atlantic salmon fed with a mix of two probiotics (*Bacillus velezensis V4* and *Rhodotorula mucilaginosa*). MDA levels, closely related to lipid peroxidation, were also reported by the same authors to be lower on probiotic-supplemented diets, contrasting with unsupplemented ones. Similarly the addition of *Bacillus subtilis* HAINUP40 was able to increase SOD present in the serum, being associated with a positive modulation of fish health and antioxidant activity (Liu et al., 2017).

Exogenous carbohydrolytic enzymes may modulate fish oxidative status and thus the enzymatic antioxidant activities by enhancing the production of microbial volatile fatty-acids and/or the production of fermented NSP fibers with antioxidant properties (Diógenes et al., 2019; Enes et al., 2012). Additionally, carbohydrases can also play a role in regulating the oxidative status of fish by mitigating the detrimental effects caused by NSPs (Fernandes et al., 2022).

Diógenes et al. (2019) reported that the incorporation of Natugrain® at a concentration of 0.1% significantly reduce the liver's vulnerability to oxidative stress induced by the addition of distillers dried grain with solubles in the diet.

In the present study, both probiotic and exogenous enzymes supplemented diets had no significant effects on CAT, GPX and GR activities. SOD was the only antioxidant enzyme modulated by dietary inclusion of FI99 and Natugrain®. Thus, diets D1 and D2 were not able to enhance SOD activity compared to the control diet, despite being comparable to EXO diet, which displayed significantly higher antioxidant enzymatic activity than the control diet. Interestingly diet D3 had the opposite effect by slightly decreasing SOD activity, thus contrasting with the other two probiotics-supplemented diets and the EXO diet. Despite this, LPO was not affected, and thus the oxidative status of the fish was not affected.

We can possibly attribute the EXO diet's higher modulation of SOD activity to the subproducts formed by the exoenzyme activity. For example, the breakdown of wheat may led to the formation of fructooligosaccharides (Campbell et al., 1997), which in turn have been reported with the potential to modulate SOD activity (Syed Raffic Ali et al., 2017). Moreover, the breakdown of hemicellulose by xylanase is known to produce subproducts such as xylooligosaccharides (XOS) (Santibáñez et al., 2021; Wang et al., 2023), that may have positive regulative effects on SOD activity, and thus, a higher antioxidant capacity of the fish. Abasubong et al. (2018) described this in blunt snout bream (*Megalobrama amblycephala*) fed with plant-based diets with 1.5% supplementation of XOS, where liver SOD activity was positively affected and MDA contents were lower, after a challenge with *Aeromonas hydrophila*.

Regardless, gut lipid peroxidation remained unaffected when the probiotic and the exoenzyme were added to the diet, indicating that the fish's antioxidant enzymatic system effectively neutralized any ROS generated by the dietary components. This in turn goes in accordance with the lack of significant differences in CAT, GPX and GR activities.

6. Conclusions and Future perspectives

Probiotics may have the capability of enhancing growth performance, by improving nutrient absorption and metabolism, modulating the intestinal microbiota, enhancing disease resistance, and modulating the oxidative status of the fish. Besides these, a higher utilization of aquafeed components may also improve aquaculture's sustainability. Consequently, there is a growing focus on the progressive study of probiotics in this context.

The capability of *Bacillus spp.* to produce spores allows the opportunity for spore supplementation before diet extrusion. This stands in contrast to typical commercial exogenous enzymes, which may face a reduction of efficacy with the elevated temperatures and pressure encountered during the extrusion process.

Despite FI99 having already shown positive effects on the growth performance of ESB fed with challenging PF-based diets, as described by Serra et al. (2019), the results of the present work allow us to conclude the following:

- Dietary incorporation of the three crescent concentrations of FI99 (6.5×10^8, 2.5×10^9 and 9.6×10^9 CFU kg^{-1} feed, respectively) had no effect on the growth performance of the fish;
- Natugrain™ supplementation at 0.04% also had no effect on the growth performance of ESB;
- FI99 and exogenous enzyme supplementations did not affect dry matter and protein ADCs, compared to control diet;
- EXO diet was able to enhance energy ADC, contrary to FI99 which lowered energy ADC in the three experimental supplementation levels;
- Digestive enzymes activities were not modulated by the inclusions of both probiotic and Natugrain™;
- Restriction of animal protein to 15% with high inclusions of PF was not able to induce any morphological changes in DI morphology;
- No effects were observed in DI histology due to probiotic and exoenzyme supplementation;
- CAT, GPX and GR activities were not affected by exoenzyme and FI99 supplementation;
- SOD activity was significantly enhanced with Natugrain™ supplementation, yet it was similar to D1 and D2 diets.

- LPO of the intestine was not affected by either probiotic or exoenzyme supplementation.

To better understand the differences in energy and dry matter ADC, between the experimental diets, fiber ADC assessment is necessary. It could also be useful to understand the carbohydrolytic activities of both the exoenzymes and FI99, as such, analysis of both β-xylanase and β-glucanase are necessary.

Microbiota analysis is required to understand if FI99 was able to colonize ESB gut.

It is also important to note that experimental diets were pelletised and not extruded. As extruded diets pass through conditions of high temperature and high pressure, exogenous enzymes cannot be included in the diet prior to this process, as enzymes may be heat-sensitive and may lose some of their efficiency (Zheng et al., 2020), as stated by Shi et al. (2016), where protease lost 62% of its activity after the extrusion of the diets. Spores, on the other hand, can tolerate high pressure and temperature conditions, thus tolerating the extrusion process (Kuebutornye et al., 2019; Nayak, 2010, 2021), as demonstrated by Niu et al. (2019), where *Bacillus licheniformis* KCCM 43270 log CFU was only reduced by 18% after the extrusion process. However, it is possible to include exogenous enzymes on extruded diets, utilizing methods such as post-extrusion coating (Dalsgaard et al., 2012), or inclusion in oils with subsequent top coating (Ogunkoya et al., 2006). As such would be interesting to observe the effects of both FI99 and the exogenous enzymes on extruded diets.

FI99 has demonstrated to have *in vitro* capability to inhibit the growth of pathogens such as *Staphylococcus aureus*, *Tenacibaculum maritimum* and *Vibrio harveyi* (Serra et al., 2019). Thus, further studies are needed to understand if FI99 has the same effects *in vivo*.

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